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Packaging Capacity and Stability of Human Adenovirus Type 5 Vectors

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Adenovirus vectors are extensively used for high-level expression of proteins in mammalian cells and are receiving increasing attention for their potential use as live recombinant vaccines and as transducing viruses for use in gene therapy. Although it is commonly argued that one of the chief advantages of adenovirus vectors is their relative stability, this has not been thoroughly investigated. To examine the genetic stability of adenovirus type 5 vectors and in particular to examine the relationship between genetic stability and genome size, adenovirus vectors were constructed with inserts of 4.88 (herpes simplex virus type 1 gB), 4.10 (herpes simplex virus type 1 gB), or 3.82 (LacZ) kb combined with a 1.88-kb E3 deletion or with a newly generated 2.69-kb E3 deletion. The net excess of DNA over the wild-type (wt) genome size ranged from 1.13 to 3.00 kb or 3.1 to 8.3%. Analysis of these vectors during serial passage in tissue culture revealed that when the size exceeded 105% of the wt genome length by approximately 1.2 kb (4.88-kb insert combined with a 1.88-kb deletion), the resulting vector grew very poorly and underwent rapid rearrangement, resulting in loss of the insert after only a few passages. In contrast, vectors with inserts resulting in viral DNA close to or less than a net genome size of 105% of that of the wt grew well and were relatively stable. In general, viruses with genomes only slightly above 105% of that of the wt were unstable and the rapidity with which rearrangement occurred correlated with the size of the insert. These findings suggest that there is a relatively tight constraint on the amount of DNA which can be packaged into virions and that exceeding the limit results in a sharply decreased rate of virus growth. The resultant strong selection for variants which have undergone rearrangement, generating smaller genomes, is manifested as genetic instability of the virus population.

For several years, human adenoviruses (Ads) have been used as vectors for expression of foreign genes in mammalian cells (1, 2, 5, 8, 11, 16, 19, 20, 25, 29, 32, 48, 50, 51, 57, 58) and studied for potential use in live viral vaccines (7, 9, 13, 20, 26, 28, 35, 37, 39–41, 57) and, more recently, for gene therapy (27, 34, 36, 42, 43, 45, 46, 52, 53, 56). A number of properties of the Ad system make it a good candidate for each of these applications, not the least of which is the extensive understanding of the structure and biology of Ads that has been gained through their use as a model system to study DNA replication, transcription, RNA processing, and protein synthesis (17). Other advantages include the ease with which the genome can be manipulated by simple recombinant DNA techniques, the high yields of virus that can be obtained and easily collected and concentrated, and the possibility of high-level expression of foreign DNA inserts (1, 19).

Construction of recombinant Ad vectors involves insertion of foreign DNA into the Ad genome, usually with compensating deletions of viral DNA. Previous work has shown that the Ad virion has the ability to package up to 105% of the wild-type (wt) genome length (15, 18), which allows for insertion of approximately 1.8 to 2.0 kb of excess DNA. By introduction of deletions in early region 1 (E1) or E3, vectors with correspondingly larger inserts can be constructed. E1 is not required for viral replication in complementing 293 cells which contain and express the left 11% of the Ad type 5 (Ad5) genome (22), and deletions of up to 2.9 kb have been made in this region (1, 18, 21, 51) to generate conditional helper-independent vectors with a capacity expected to be up to approximately 4.7 to 4.9 kb. The E3 region has been shown to be nonessential for viral replication in any normally permissive cells and can be deleted to produce nonconditional helper-independent vectors (3, 24). The maximum possible size of nonlethal deletions in the E3 region has not been determined, but they presumably cannot extend into the essential virion structural genes, those for pVIII and fiber, that flank this region. Many nonconditional helper-independent vectors have been developed by utilizing a 1.88-kb deletion in this region. Vectors of this type should have a capacity for inserts of approximately 3.7 to 3.9 kb (3).

Application of Ad vectors to gene therapy requires a more thorough examination of vector stability and packaging capacity than has yet been done. It is generally assumed that Ad vectors are relatively stable and can package DNA up to 105% of the size of the wt genome. To examine the genetic stability of Ad5 vectors and in particular to correlate genetic stability and genome size, a series of Ad vectors were constructed with E3 substitutions representing net insertions of up to 8.3% of the Ad5 genome. When the structures of these recombinant viruses were analyzed after serial passages in 293 cells, it was found that stability correlated with net insert size and vectors with the largest inserts rearranged extremely rapidly.

MATERIALS AND METHODS

Construction of recombinant plasmids. Enzymes used for recombinant DNA manipulations were purchased from Boehringer-Mannheim, Inc. (Laval, Quebec, Canada); New England BioLabs (Beverly, Mass.); or Bethesda Research Laboratories (Burlington, Ontario, Canada) and used in accordance with the supplier's recommendations. Plasmids

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were constructed by using standard protocols (47). Electroporation (12) was used to transform *Escherichia coli* DH5 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) with newly constructed plasmids. Plasmid DNA was prepared by the alkaline lysis method (4) and purified by CsCl-ethidium bromide density gradient centrifugation.

Cells and viruses. Cell culture media and reagents were obtained from GIBCO Laboratories (Grand Island, N.Y.). Ad vectors were titered and passaged on 293 cells, which constitutively express the left 11% of the Ad5 genome (22). The 293 cells were grown in monolayer in F-11 minimum essential medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2.5 μg of amphotericin per ml, along with 10% newborn calf serum for cell maintenance or 5% horse serum for virus infection. KB cells grown in spinner culture were maintained in Joklik's modified medium supplemented with antibiotics as described above and with 10% horse serum.

For one-step growth curves, KB cells were grown to a density of 2×10^5/ml, centrifuged, and resuspended in 1/10 of the volume of the original medium. Virus was then added at a multiplicity of infection (MOI) of 20 and allowed to adsorb for 1 h at 37°C with shaking. The cells were then returned to the original volume with 50% fresh medium and 50% original medium. At various times postinfection, 4-ml aliquots were taken, 0.5 ml of glycerol was added, and samples were stored at −70°C for assays of infectious virus by plaque titration.

Construction and growth of recombinant viruses. Recombinant viruses were isolated by cotransfection of 293 cells with appropriate plasmids (23). After 8 to 10 days, plaques were isolated and expanded and viral DNA was analyzed by restriction enzyme digestion as described previously (19, 24). Candidate viruses were then plaque purified once, and for stability studies, vectors were passaged by starting with medium from cells infected for viral DNA analysis after the first plaque purification. Semiconfluent monolayers of 293 cells in 60-mm-diameter dishes were infected with 0.5 ml of medium from each previous passage (an MOI of approximately 40), virus was allowed to adsorb for 0.5 h, and then the medium was replaced. Cells and medium were harvested when the cytopathic effect was complete, usually within 2 to 3 days postinfection.

^{32}P labelling and extraction of viral DNA. Semiconfluent monolayers of 293 cells in 60-mm dishes were infected with virus from passages to be analyzed, and at 24 h postinfection, medium was removed and replaced with 1 ml of phosphate-free 199 medium containing 5% horse serum and 25 μCi of $^{32}P_i$ (Du Pont de Nemours & Co. Inc., Wilmington, Del.) per ml. After incubation of the infected cells for a further 6 h, they were harvested and DNA was extracted (19). Viral DNA was then digested with appropriate restriction enzymes and electrophoresed through 1% agarose gels, the gels were dried, and DNA bands were visualized by autoradiography.

^{35}S labelling of proteins. Semiconfluent monolayers of 293 cells in 60-mm dishes were infected at an MOI of 20. At 22 h postinfection, the medium was removed and replaced with 3 ml of methionine-free 199 medium supplemented with minimal essential medium vitamin solution (GIBCO), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2), 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2% dialyzed fetal calf serum and incubated at 37°C for 45 min. This medium was then removed and replaced with 1 ml of methionine-free medium containing 50 μCi of [^{35}S]methionine (Trans ^{35}S-label [1,000 Ci/mmol]; ICN Biomedicals Inc., Irvine, Calif.). The cells were labelled for 1 h at 37°C, washed with ice-cold phosphate-buffered saline; and harvested by scraping in 0.5 ml of ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 10 μg of aprotinin [Sigma, St. Louis, Mo.] per ml, 0.2 mM phenylmethylsulfonyl fluoride). The cell extracts were then vortexed and centrifuged for 30 min at 4°C and 30,000 × *g* and the supernatant was collected for analysis. Samples of cell extract were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 8.0% gels, the gels were dried, and protein bands were visualized by autoradiography. Densitometry was performed with an LKB Ultroscan XL Enhanced Laser Densitometer.

RESULTS

A vector with a 39-kb genome is genetically unstable. As one of a series of Ad vectors expressing herpes simplex virus type 1 (HSV-1) glycoproteins, an Ad5 vector (AdgB10) with a 4.88-kb insert containing the HSV-1 gB gene in combination with an E3 deletion of 1.88 kb was constructed. The structure of this vector is illustrated in Fig. 1a. AdgB10 has a net insertion of 3.0 kb, representing 8.3% of the wt Ad5 genome, or approximately 1.2 kb more excess DNA than has been cloned in an Ad5 vector previously (1, 15). Initial findings indicated that AdgB10 replicated very inefficiently in early passages on 293 cells, requiring two to three times longer to induce a cytopathic effect following a typical infection of 293 cells compared with the wt virus or other vectors. After a few passages, a virus population which was able to replicate more rapidly than the original clone was obtained from the original AdgB10 plaque isolate. However, analysis of viral DNA from consecutive passages (Fig. 1b) indicated that the virus stock had undergone rapid rearrangement as determined by loss of the diagnostic 8.66-kb *Hin*dIII B′ fragment containing the HSV-1 gB insert (solid arrowhead in Fig. 1b). By passage 7, the viral DNA preparations no longer contained any detectable 8.66-kb *Hin*dIII fragment, which was replaced by a smaller fragment (open arrowhead in Fig. 1b) of about 3.2 kb that was first detected at passage 5. Thus, the increased growth rate seen as AdgB10 was serially passaged through 293 cells appeared to result from generation and outgrowth of a deletion variant with a net genome size approximately 95% of that of the wt. The faint bands seen between *Hin*dIII fragments A and C in the various passages shown in Fig. 1b are likely due to the presence of other deletion variants in the viral population. In two additional independent serial passages with AdgB10, two different rearrangements of the diagnostic 8.66-kb *Hin*dIII fragment were obtained, one in which a new fragment of 3.0 kb appeared at passage 2 and another in which a fragment of 3.4 kb was apparent by passage 4 (Fig. 1c). Since the *Hin*dIII B fragment containing just the 1.88-kb deletion results in a B′ fragment of 3.78 kb, it is apparent that in all three of these serial passages, AdgB10 had undergone a rearrangement which resulted in loss of all or part of the gB insert, as well as some of the surrounding Ad DNA. Because vectors made previously with smaller net insertions (less than 5.0% of the genome) had not demonstrated such severe instability (unpublished data), it seemed likely that the rapid appearance of rearrangements was due to the size of the AdgB10 genome. Since the packaging capacity of Ad virions is likely to be a limiting factor in the cloning and expression of some genes, we decided to develop vectors with an

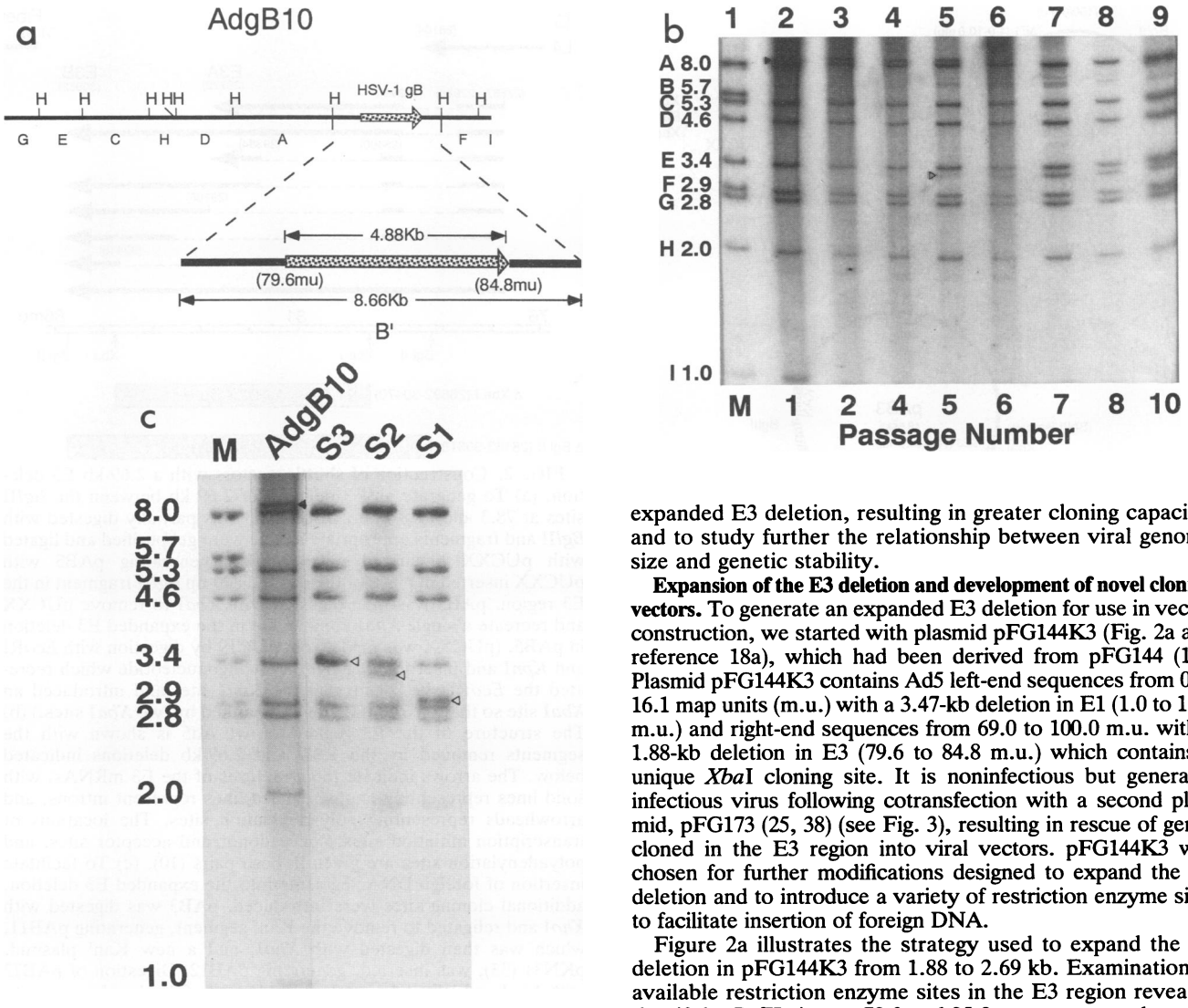
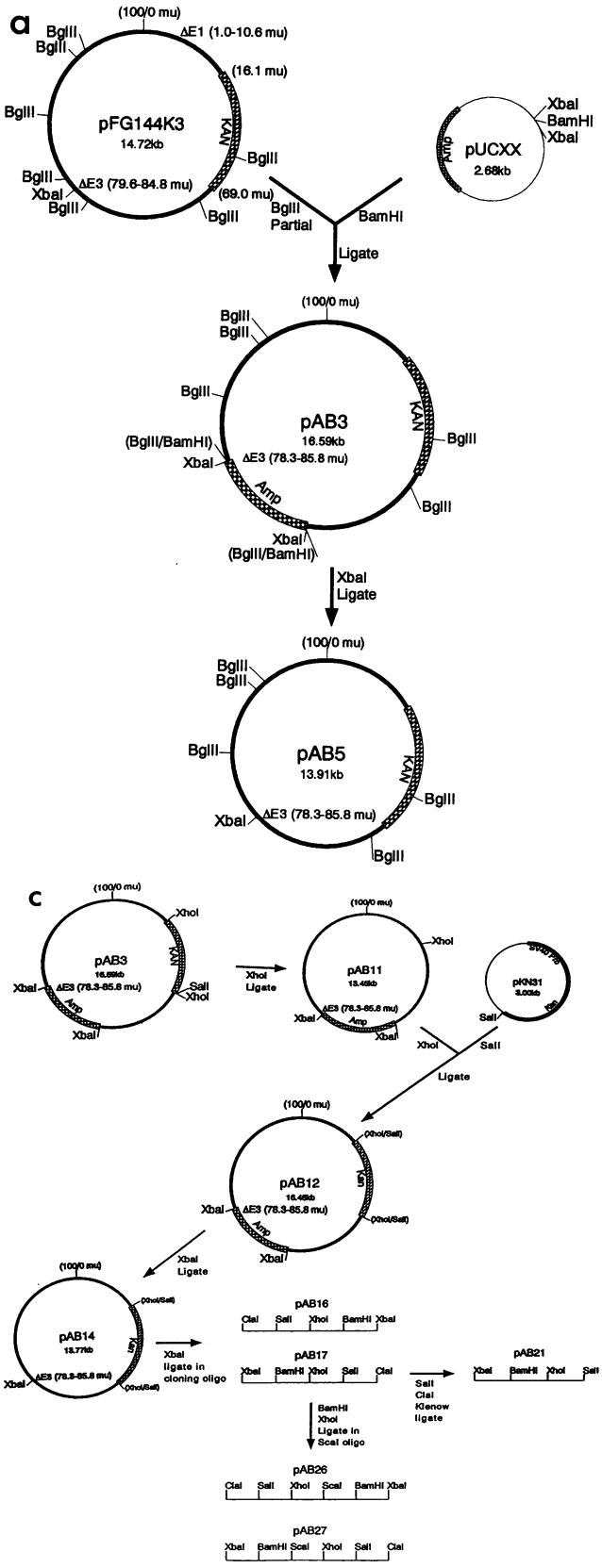


FIG. 1. Structure and genetic stability of AdgB10. (a) AdgB10 contains a 4.88-kb insert spanning the HSV-1 gB gene combined with an E3 deletion of 1.88 kb. *Hin*dIII restriction sites (H) are indicated above the line, and *Hin*dIII restriction fragments are labelled by letter below the line. Fragments A, C, D, E, F, G, and I are identical to wt Ad5 *Hin*dIII fragments, and B′ is a diagnostic 8.66-kb fragment containing the 4.88-kb HSV-1 gB insert. Map units (mu) refer to positions on the Ad5 genome. The solid bars represent Ad5 sequences, and the stippled bars represent HSV-1 gB sequences. (b) *Hin*dIII digestion patterns for viral DNA from various passages of AdgB10. 293 cells were infected with virus from passages 1, 2, 4, 5, 6, 7, 8, and 10 for 24 h and then labelled with $^{32}P_i$ from 24 to 30 h postinfection. Infected cell DNA was extracted, digested with *Hin*dIII, and separated by electrophoresis on a 1% agarose gel. The gel was then dried, and bands were visualized by autoradiography. Lane 1 contained wt Ad5 DNA digested with *Hin*dIII. Fragment sizes are indicated to the left in kilobases. Lanes 2 to 9 contained the passages of AdgB10 indicated below the lanes. In lane 2, the 8.66-kb fragment containing gB is indicated by a solid arrowhead. Over passages 2 to 5, the intensity of this fragment was reduced and replaced by a rearranged fragment of 3.2 kb, indicated by an open arrowhead in passage 5, lane 5. (c) *Hin*dIII digestion patterns for viral DNA representing the three rearrangements detected in three independent serial passages carried out with AdgB10. 293 cells were infected with virus from the initial plaque purification of AdgB10, passage 10 series 1, passage 10 series 2, and passage 8 series 3, for 24 h and then labelled with $^{32}P_i$ from 24 to 30 h postinfection. Infected cell DNA was extracted, digested with *Hin*dIII, and separated by electrophoresis on a 1% agarose gel. The gel was then dried, and bands were visualized by autoradiography. Lane M contained wt Ad5 DNA digested with *Hin*dIII. Fragment sizes are indicated to the left in kilobases. The remaining lanes contained the series of AdgB10 indicated above. The 8.66-kb fragment containing gB is indicated by a solid arrowhead, and the rearranged fragments are indicated with open arrowheads. In series 3, a rearranged fragment of approximately 3.4 kb was present which ran as a doublet with *Hin*dIII fragment E, in series 2, a rearranged fragment of approximately 3.2 kb was seen; and in series 1, a rearranged fragment of approximately 3.0 kb was observed.

expanded E3 deletion, resulting in greater cloning capacity, and to study further the relationship between viral genome size and genetic stability.

Expansion of the E3 deletion and development of novel cloning vectors. To generate an expanded E3 deletion for use in vector construction, we started with plasmid pFG144K3 (Fig. 2a and reference 18a), which had been derived from pFG144 (14). Plasmid pFG144K3 contains Ad5 left-end sequences from 0 to 16.1 map units (m.u.) with a 3.47-kb deletion in E1 (1.0 to 10.6 m.u.) and right-end sequences from 69.0 to 100.0 m.u. with a 1.88-kb deletion in E3 (79.6 to 84.8 m.u.) which contains a unique *Xba*I cloning site. It is noninfectious but generates infectious virus following cotransfection with a second plasmid, pFG173 (25, 38) (see Fig. 3), resulting in rescue of genes cloned in the E3 region into viral vectors. pFG144K3 was chosen for further modifications designed to expand the E3 deletion and to introduce a variety of restriction enzyme sites to facilitate insertion of foreign DNA.

Figure 2a illustrates the strategy used to expand the E3 deletion in pFG144K3 from 1.88 to 2.69 kb. Examination of available restriction enzyme sites in the E3 region revealed that if the *Bgl*II sites at 78.3 and 85.8 m.u. were used, an E3 deletion of 2.69 kb could be generated that would not delete the essential virion structural genes, i.e., those for pVIII and fiber (Fig. 2b). Since pFG144K3 contains a total of seven *Bgl*II sites, partial digestion with *Bgl*II was performed and fragments of the appropriate size were gel purified and ligated with Amp^r plasmid pUCXX linearized with *Bam*HI. Restriction enzyme analysis was used to screen for the desired plasmid, pAB3, which contains pUCXX inserted, by fusion of *Bgl*II-*Bam*HI complementary ends, in place of the



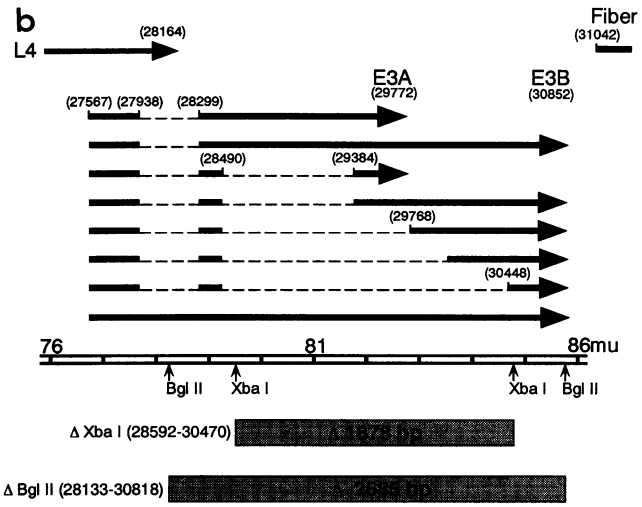


FIG. 2. Construction of shuttle vectors with a 2.69-kb E3 deletion. (a) To generate an E3 deletion of 2.69 kb between the *Bgl*II sites at 78.3 and 85.8 m.u., pFG144K3 was partially digested with *Bgl*II and fragments appropriate in size were gel purified and ligated with pUCXX linearized with *Bam*HI, generating pAB3 with pUCXX inserted in place of the deleted 800-bp *Bgl*II fragment in the E3 region. pAB3 was then digested with *Xba*I to remove pUCXX and recreate a single *Xba*I cloning site in the expanded E3 deletion in pAB5. (pUCXX was made from pUC19 by digestion with *Eco*RI and *Kpn*I and insertion of a synthetic oligonucleotide which recreated the *Eco*RI site, destroyed the *Kpn*I site, and introduced an *Xba*I site so that the *Bam*HI site was flanked by two *Xba*I sites.) (b) The structure of the E3 region for wt Ad5 is shown with the segments removed by the 1.88- and 2.69-kb deletions indicated below. The arrows indicate the structures of the E3 mRNAs, with solid lines representing exons, dashed lines represent introns, and arrowheads representing polyadenylation sites. The locations of transcription initiation sites, splice donor and acceptor sites, and polyadenylation sites are given in base pairs (10). (c) To facilitate insertion of foreign DNA segments into the expanded E3 deletion, additional cloning sites were introduced. pAB3 was digested with *Xho*I and religated to remove the Kan^r segment, generating pAB11, which was then digested with *Xho*I, and a new Kan^r plasmid, pKN31 (33), was inserted, generating pAB12. Digestion of pAB12 with *Xba*I and religation resulted in removal of the Amp^r segment to create pAB14. Finally, the polycloning sites indicated were introduced into the *Xba*I site of pAB14, generating pAB16, pAB17, pAB21, pAB26, and pAB27. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, and hatched bars represent Amp^r and Kan^r segments.

deleted 800-bp *Bgl*II fragment in the E3 region. pAB3 was then digested with *Xba*I and ligated to remove pUCXX sequences, generating pAB5. This step recreated a single *Xba*I cloning site in the expanded E3 deletion while leaving 20 bp of pUCXX DNA. The structure of the region spanning E3 for wt Ad5 and the deletions present in pFG144K3 and pAB5 are shown in Fig. 2b.

To facilitate insertion of foreign DNA segments into shuttle plasmids containing the expanded E3 deletion, additional cloning sites were introduced by the procedures outlined in Fig. 2c. So that *Sal*I and *Xho*I sites could be used as cloning sites, the Kan^r segment of pAB3 was removed by *Xho*I digestion and religation, generating pAB11, and a new Kan^r plasmid, pKN31, was inserted into the *Xho*I site of pAB11 by fusion of *Sal*I-*Xho*I complementary ends, generating pAB12. The Amp^r segment was then removed from the E3 region of pAB12 to generate pAB14. Various cloning

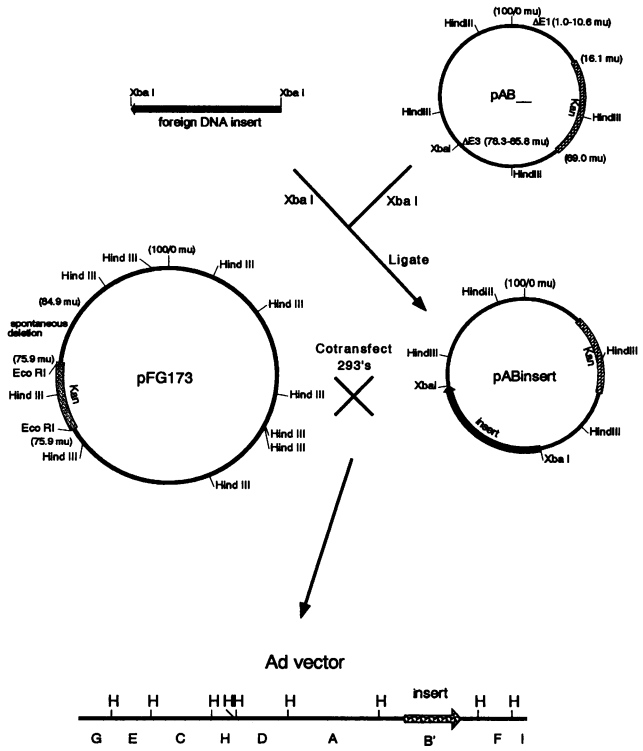


FIG. 3. Rescue of inserts into the E3 region with E3 shuttle vectors. The general strategy used to introduce foreign DNA inserts into either the 1.88- or 2.69-kb E3 deletions for rescue into virus is illustrated. The foreign DNA fragment insert was isolated with appropriate flanking restriction enzyme sites (*Xba*I in the example shown) for insertion into the E3 deletion of one of the pAB series of plasmids, generating a recombinant plasmid with the insert in the E3 parallel orientation. The recombinant plasmid, pABinsert, was then cotransfected with pFG173 (25, 38) into 293 cells, and infectious virus was generated by in vivo recombination.

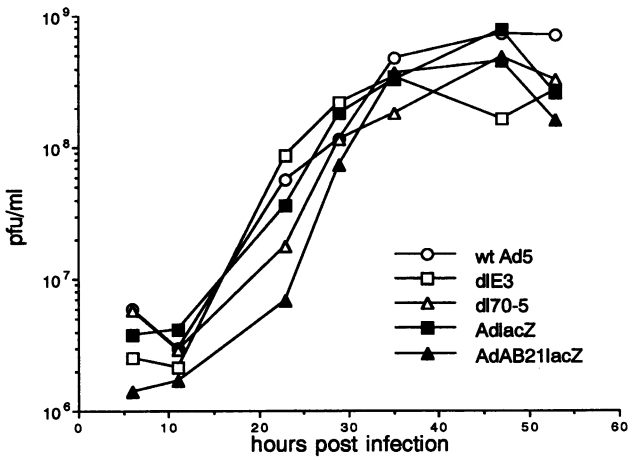


FIG. 4. Growth kinetics of viruses with the expanded E3 deletion. The growth properties of viruses with the 2.69-kb E3 deletion were studied and compared with those of wt Ad5 and viruses containing the 1.88-kb deletion by generating one-step growth curves. Spinner cultures of KB cells were infected at an MOI of 20 with wt Ad5, *dl*E3, *dl*70-5, AdlacZ, or AdAB21lacZ, samples were taken at various times postinfection, and their titers were determined on 293 cells. *dl*70-5 contains the 2.66-kb E3 deletion, *dl*E3 contains the 1.88-kb E3 deletion, AdlacZ contains the *lacZ* gene flanked by the SV40 promoter and poly(A) sequences inserted in the 1.88-kb E3 deletion, and AdAB21lacZ contains the *lacZ* gene with SV40 poly(A) sequences inserted in the 2.66-kb E3 deletion. The *lacZ* inserts were introduced in the E3 parallel orientation.

sites were then introduced into the *Xba*I site of pAB14, generating shuttle vectors pAB16, pAB17, pAB21, pAB26, and pAB27.

All of the E3 deletion plasmids were sequenced in the region of the expanded deletion to confirm that they had the correct structures. The 2.69-kb E3 deletion present in the plasmids described above removed two additional elements that were not removed by the original 1.88-kb deletion. The 1.88-kb deletion removed the major parts of all E3 messages while leaving intact the E3 promoter and 5′ initiation site, the first set of E3 5′ and 3′ splice sites, and the E3 and L4 polyadenylation sites (3). As illustrated in Fig. 2b, the 2.69-kb E3 deletion, in addition to removing the major parts of all E3 messages, removed the first E3 3′ splice acceptor site and the L4 polyadenylation site (10).

Four of the plasmids described, pAB5, pAB14, pAB16, and pAB17, were rescued into virus by cotransfection with pFG173 (25, 38) into 293 cells, as illustrated in Fig. 3, generating viruses *dl*70-2, *dl*70-4, *dl*70-5, and *dl*70-6, respectively. These viruses were shown to have the expected structures by restriction analysis (data not shown).

As mentioned above, the newly created E3 deletion in the pAB series of shuttle plasmids lacked transcription signals that were present in earlier vectors with the 1.88-kb deletion. Therefore, it was of interest to analyze the growth properties of viruses containing this larger deletion. To do so, one-step growth curves were determined for several viruses containing either no deletion of E3 sequences (wt Ad5), the 1.88-kb deletion (*dl*E3), the 2.69-kb deletion (*dl*70-5), or a β-galactosidase gene inserted in the 1.88-kb deletion (AdlacZ) or in the 2.69-kb deletion (AdAB21lacZ) (Fig. 4). Spinner cultures of KB cells were infected at an MOI of 20, samples were taken at various times postinfection, and their titers were determined on 293 cells. The results shown in Fig. 4 indicated a somewhat lengthened eclipse phase for viruses with the 2.69-kb E3 deletion, but in other experiments this lag in virus replication was less pronounced (data not shown). Final yields of viruses with the 2.69-kb E3 deletion, with or without inserts, were always in the range of those obtained for wt Ad5 (data not shown).

To assess the effect of removal of the L4 polyadenylation site on expression of the L4 products, we compared the levels of 100K synthesized in 293 cells infected with wt Ad5, *dl*309 (31), *dl*E3, *dl*70-5, AdlacZ, or AdAB21lacZ (Fig. 5). 293 cells were infected at an MOI of 20 and labelled with [^{35}S]methionine from 23 to 24 h postinfection, cell extracts were harvested, and samples were run on SDS–8.0% PAGE gels. The results shown in Fig. 5 indicated that there was, at most, a one-third reduction in the rate of synthesis of 100K for the viruses analyzed, suggesting that removal of the L4 polyadenylation signal by the 2.69-kb E3 deletion had not greatly affected the expression of L4 products. It is of interest that the level of fiber was reduced relative to those of other late proteins in vectors containing inserts in either the 1.88- or 2.69-kb E3 deletion. This reduction, which has been noted previously (38), seems to correlate inversely with the level of expression of the insert but does not appear to affect viral replication. Densitometry was used to more accurately determine the levels of 100K and fiber expressed by the various vectors. In Table 1, the results of two such analyses

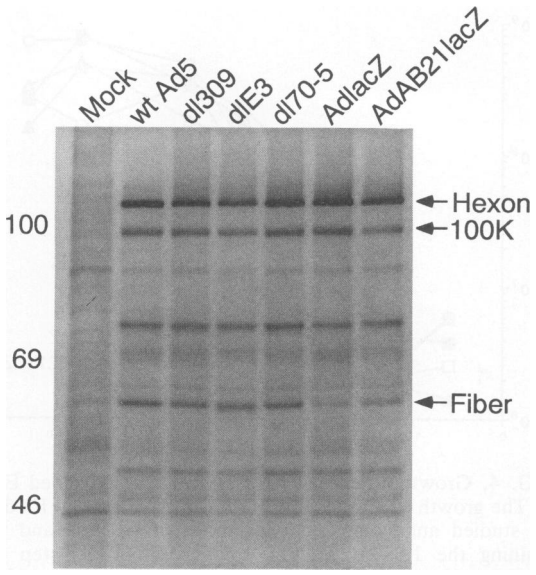


FIG. 5. 100K expression in viruses with the expanded E3 deletion. The rates of synthesis of 100K, an L4 product, for viruses with the 2.69-kb E3 deletion were determined and compared with those of wt Ad5 and viruses with the 1.88-kb deletion. 293 cells were either mock infected or infected at an MOI of 20 with wt Ad5, *dl*309, *dl*E3, *dl*70-5, AdlacZ, or AdAB21lacZ for 23 h and then labelled with [^{35}S]methionine from 23 to 24 h postinfection. Cell extracts were then prepared, and samples were separated by SDS-PAGE on an 8.0% gel. The gel was then dried, and bands were visualized by autoradiography. The relative levels of 100K present in the samples run on this gel were determined by densitometric analysis (Table 1). The lanes contained the samples indicated, and molecular weight markers are indicated on the left in thousands. *dl*70-5 carries the 2.66-kb E3 deletion, *dl*E3 has the 1.88-kb E3 deletion, AdlacZ contains the *lacZ* gene flanked by the SV40 promoter and poly(A) sequences inserted in the 1.88-kb E3 deletion, and AdAB21lacZ contains the *lacZ* gene with SV40 poly(A) sequences inserted in the 2.66-kb E3 deletion. The *lacZ* inserts were introduced in the E3 parallel orientation.

TABLE 1. Synthesis of 100K and fiber in cells infected with E3 deletion viruses[a]

Expt and virus	Size of E3 deletion (kb)	Expression level (%)[b]	
		100K	Fiber
1			
wt Ad5	0	100	100
*dl*309	Del-sub[c]	113	100
*dl*E3	1.88	102	135
*dl*70-5	2.69	98	87
AdlacZ	1.88	95	41
AdAB21lacZ	2.69	67	39
2			
*dl*309	Del-sub[c]	100	100
*dl*70-4	2.69	113	112
*dl*70-5	2.69	105	118
AdHTLV1gag2L	2.69	92	30
Ad5-luc11	2.69	68	38
Ad5-luc11L	2.69	86	92

[a] Cells infected with the indicated viruses were labelled with [^{35}S]methionine and cell extracts were prepared and run on polyacrylamide gels. The levels of 100K and fiber were determined by densitometric analysis.

[b] For each virus, the levels of 100K and fiber were determined relative to that of hexon and then expressed as a percentage of the wt Ad5 level (experiment 1) or the *dl*309 level (experiment 2).

[c] The deletion and substitution present in *dl*309 (31).

are shown; the first is from the autoradiogram presented in Fig. 5, and the second is from a gel on which recombinants with different inserts in the 2.69-kb E3 deletion were similarly analyzed (data not shown). In experiment 2 in Table 1, AdHTLV1gag2L is an Ad vector containing a 4.0-kb insert consisting of the human T-cell leukemia virus type I *gag* region and an Amp^r gene in the E3 antiparallel orientation (10a), and Ad5-luc11 and Ad5-luc11L are vectors containing a 1.7-kb insert of the luciferase gene in the E3 parallel and antiparallel orientations, respectively (37a). Densitometry revealed that viruses with the 1.88- or 2.69-kb E3 deletion or an insert in the 1.88-kb deletion expressed wt levels of 100K. In vectors that contain an insert in the 2.69-kb E3 deletion, the level of 100K varied from 92 to 67% relative to that in the wt, depending on the insert and its orientation. In viruses with the 2.69-kb deletion and no insert, it is possible that the E3b polyadenylation site can functionally substitute for the L4 polyadenylation signal (Fig. 2b). Differences in 100K levels seen with vectors carrying different E3 insertions may be due to the presence or absence of sequences in inserted genes that can act as fortuitous polyadenylation signals for L4 transcripts. The reduced levels of 100K and fiber synthesis seen with some vectors appear not to have significant effects on infectious virus yields, probably because viral late proteins are normally made in excess during viral replication. In a recent report by Ranheim et al. (44), a 3.0-kb E3 deletion was generated that also removed the L4 polyadenylation signal; it was also found that viruses with this deletion replicate to the same final titers as wt Ad5.

Construction and characterization of additional vectors. With the verification that the growth kinetics and expression of L4 products in vectors containing the expanded 2.69-kb E3 deletion were not significantly altered, it was decided to utilize the pAB shuttle vectors in further analyses of vector stability. By substituting inserts of various sizes for the 1.88- and 2.69-kb E3 deletions, vectors with a range of net genome sizes were easily generated. The DNA segments introduced into the two E3 deletions were a 4.88-kb fragment containing the transcriptional and translational information for HSV-1 gB, a 4.10-kb fragment containing the coding sequences for HSV-1 gB fused to the simian virus 40 (SV40) promoter, and a 3.82-kb fragment containing the coding sequences for LacZ flanked by the SV40 promoter and poly(A) signal. The inserts were introduced into shuttle plasmids containing the 1.88- or 2.69-kb E3 deletion and rescued into virus by cotransfection with pFG173 into 293 cells as outlined in Fig. 3.

Viral plaques obtained from cotransfections were isolated and plaque purified once, and viral DNA was analyzed by restriction enzyme digestion to ensure that the desired recombinant was obtained. The vectors constructed (Fig. 6) represent a series of viruses with net genome sizes ranging from 103.1 to 108.3% of the wt Ad5 genome length (Table 2).

As discussed previously, AdgB10, with a genome size of 108.3% relative to the wt, grew very inefficiently and underwent rapid rearrangement, resulting in the outgrowth of deletion variants able to replicate more rapidly. Detection of three different rearranged forms after three independent serial passages of the AdgB10 isolate (Fig. 1c) indicated that the appearance of rearrangements was not due to selection for a single variant preexisting in the virus population. Rather, AdgB10 was able to generate a number of different variants, each of which replicated sufficiently well to predominate after a few passages.

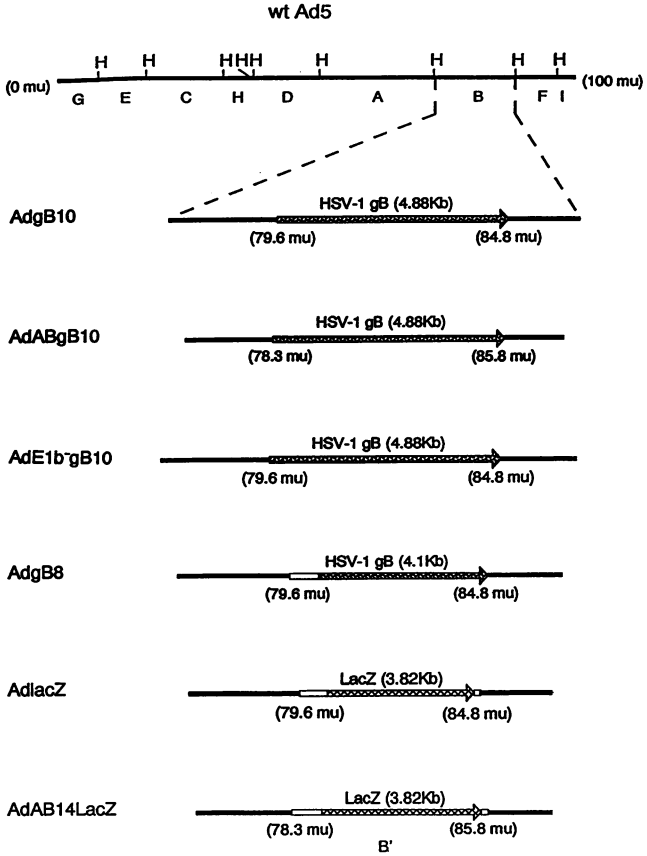


FIG. 6. Structures of viruses used in stability study. The structures of the viruses used in the analysis of vector stability are shown. *Hin*dIII restriction fragments are indicated by letter, and the *Hin*dIII B' fragment of each virus is expanded, showing the DNA segment inserted and the E3 deletion used. AdABgB10, AdgB8, AdLacZ, and AdAB14LacZ were all constructed by using basically the strategy outlined in Fig. 3. AdE1b⁻gB10 was created by using a different strategy and also contains a deletion of E1b sequences from 5.5 to 9.3 m.u. AdE1b⁻gB10 was created by digesting pFGdx1gB with *Spe*I and ligating it with Ad5*dl*55 viral DNA (18a) digested with *Spe*I and *Eco*RI (the latter enzyme was used to reduce the infectivity of parental *dl*55 DNA). The ligated DNA was then used to transfect 293 cells, and viral plaques were picked, expanded, and analyzed by restriction digestion to identify the desired E1b⁻ recombinants. Map units (mu) refer to Ad5 sequences. The solid bars represent Ad5 sequences, hatched bars represent inserts of HSV-1 gB or LacZ, as indicated, and open bars represent SV40 sequences.

To obtain a vector similar to AdgB10 but with a smaller net genome size, AdABgB10 was constructed by combining the 4.88-kb HSV-1 gB insert with the 2.69-kb E3 deletion (Fig. 7a). AdABgB10 has a net genome size of 106.1% of that of wt Ad5 and was found to replicate normally and induce a cytopathic effect following an incubation time similar to that required for wt Ad5 and other vectors during a typical infection of 293 cells. When AdABgB10 was serially passaged through 293 cells, it was found to be significantly more stable than AdgB10, taking severalfold more passages for rearrangements to appear. Figure 7b shows one of three independent stability assays performed with AdABgB10, in which the various passages shown were analyzed by restriction digestion with *Hin*dIII and *Xba*I. *Hin*dIII-*Xba*I double digestion was used in this case, since digestion with *Hin*dIII alone resulted in a B' fragment of 7.86 kb which ran as a doublet with *Hin*dIII fragment A, making analysis difficult. The 4.88-kb gB insert which was excised by *Xba*I is indicated by a solid arrow in Fig. 7b (lane 2). In this series, two deletion variants were detected, one at passage 14, when a fragment of 2.7 kb was first observed, and the second at passage 22, when a fragment of 1.9 kb appeared. These new fragments (indicated by open arrows in Fig. 7b) became more intense during successive passages as the 4.88-kb gB fragment became less intense. The first variant detected at passage 14 appeared to have deleted all or part of the gB insert and possibly some essential Ad sequences, thus generating a defective virus unable to completely overtake the viral population. Following low-MOI infections with virus from passage 14, only the parental AdABgB10 digest pattern was detected, consistent with elimination of a defective helper-dependent variant from the population (data not shown). The second variant detected in passage 22, which also appeared to have deleted gB insert sequences, was not defective, since in low-MOI infections with virus from passage 22 the variant pattern was detected, with complete loss of the 4.88-kb gB fragment (data not shown). In the other two stability assays done with AdABgB10, two different rearrangements were observed, which were first detected at passages 9 and 10 (Table 2). These rearrangements did not involve the gB insert in E3 but appeared to result in loss of essential sequences in *Hin*dIII fragment C (m.u. 17.4 to 32.2). That these variants represented defective, helper-dependent viruses was confirmed by further analysis, since they were eliminated following low-MOI infections and only the parental AdABgB10 digest pattern was detected (data not shown).

The detection of rearrangements involving *Hin*dIII fragment C in two of the stability assays performed with AdABgB10 seemed to suggest that the important factor causing the rearrangement of the vectors was the net genome size and not the insert or the flanking Ad sequences. To confirm that the difference in stability between AdgB10 and AdABgB10 was not due to the difference in the context of the

TABLE 2. Stability of Ad5 vectors having genomes of various sizes[a]

Virus	Deletion in E3 (kb)	Insert size (kb)	Net genome size (%)[b]	Rearrangement(s) detected, passage no(s).
Ad5			100	
AdgB10	1.88	4.88	108.3	S1, p2[c]; S2, p5; S3, p4
AdgB8	1.88	4.10	106.2	S1, p8; S2, p7; S3, p9
AdABgB10	2.69	4.88	106.1	S1, p14; S2, p10[d]; S3, p9[d]
AdlacZ	1.88	3.82	105.4	S1, p7; S2, p6
AdE1b⁻gB10[e]	1.88	4.88	104.6	No change to passage 20
AdAB14lacZ	2.69	3.82	103.1	S1, p19; S2, p20

[a] DNAs from Ad5 vectors serially passaged in 293 cells were analyzed by restriction enzyme digestion and gel electrophoresis.

[b] Net genome size represents the insert size minus the deletion of E3 sequences divided by the wt genome length (35,935 bp) and is expressed as a percentage.

[c] Novel bands were detected at the indicated passages (p), indicating the appearance of viruses with rearranged genomes. S1 and S2, etc., represent independent serial passages starting from the same plaque isolate.

[d] Rearrangements involved loss of sequences in *Hin*dIII fragment C.

[e] Vector with deletions in both E1 and E3.

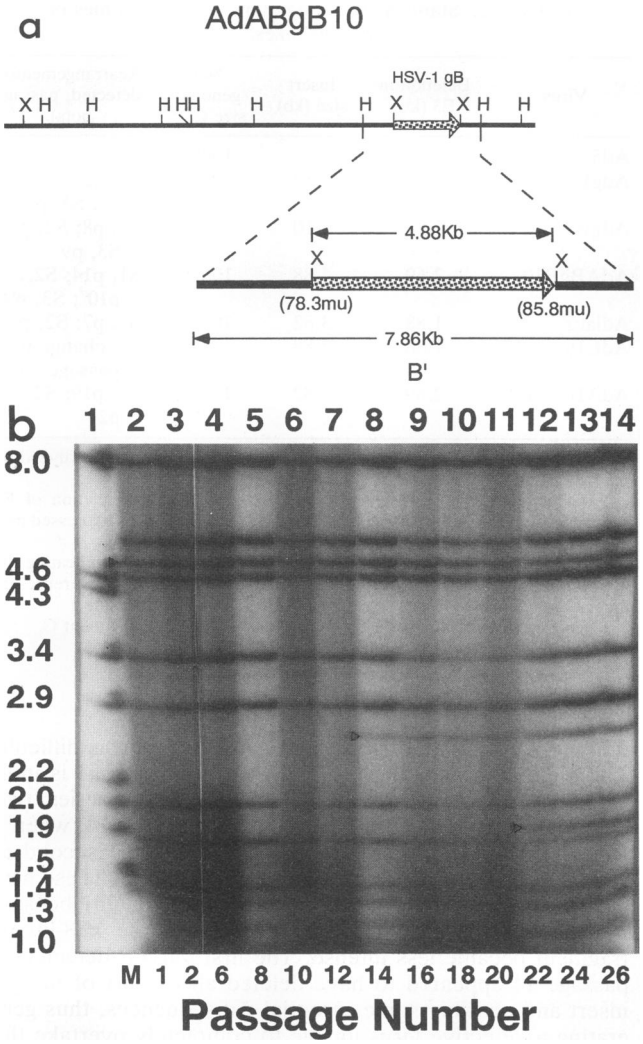


FIG. 7. Structure and genetic stability of AdABgB10. (a) AdABgB10 contains a 4.88-kb insert combined with a 2.69-kb E3 deletion. The 4.88-kb insert contains the transcriptional and translational information for HSV-1 gB. The *Hin*dIII and *Xba*I restriction sites are indicated. Map units (mu) refer to Ad5 sequences. The solid bars represent Ad5 sequences, and the stippled bars represent HSV-1 gB sequences. (b) *Hin*dIII-*Xba*I restriction analysis of passages of AdABgB10 are shown. 293 cells were infected with virus from various passages, as indicated at the bottom, and labelled with $^{32}P_i$ from 24 to 30 h postinfection. Infected cell DNA was extracted, digested with *Hin*dIII and *Xba*I, and separated by electrophoresis on a 1% agarose gel, the gel was dried, and bands were visualized by autoradiography. Lane 1 contained wt Ad5 DNA digested with *Hin*dIII and *Xba*I. Fragment sizes are indicated to the left in kilobases. Lanes 2 to 14 contained the passages of AdABgB10 indicated below the lanes. In lane 2, the 4.8-kb gB fragment is indicated with a solid arrowhead. Rearrangement was first detected at passage 14, when a fragment of 2.8 kb (open arrow, lane 8) began to appear, and a second rearrangement was detected at passage 22 (open arrow, lane 12), when a 1.9-kb fragment became apparent.

E3 region containing the gB insert in these vectors and to generate a vector with a smaller net genome size, AdE1b$^-$gB10 was created. AdE1b$^-$gB10 contained the same structure flanking the E3 region (4.88-kb HSV-1 gB insert in the 1.88-kb E3 deletion) as AdgB10 but also had a deletion of E1b sequences from 5.5 to 9.3 m.u., resulting in a net genome size of 104.6% of the wt Ad5 genome (Fig. 6 and Table 2). When viral DNA from serial passages of AdE1b$^-$gB10 was analyzed, no rearrangements were detected to passage 20, indicating that the net genome size of AdgB10, rather than the sequences in the vicinity of the insert, was the cause of its genetic instability.

The results discussed above suggested that as the net genome sizes of the vectors decreased, the viruses became increasingly genetically stable. To analyze the stability of viruses with different inserts in the E3 deletions, the structures of three additional vectors, AdgB8, AdlacZ, and AdAB14lacZ, were analyzed following serial passage. AdgB8, which had a net genome size of 106.2% of the wt Ad5 genome (Fig. 6) was found to undergo rearrangements which appeared to involve the gB insert and surrounding Ad sequences, first detected at passages 8, 7, and 9 in the three independent stability assays performed with this vector (Table 2). AdgB8, whose genome size is very close to that of AdABgB10, was found to have essentially the same degree of stability. AdlacZ and AdAB14lacZ (Fig. 6) had net genome sizes of 105.4 and 103.1% of the wt Ad5 genome, respectively. AdlacZ was found to undergo rearrangements first detected at passages 7 and 6 in the two stability assays carried out with this vector (Table 2). The stability of AdAB14lacZ was found to be dramatically increased, with rearrangements not being detected until passages 19 and 20 (Table 2). The deletion variants detected for these two vectors appeared to have lost insert sequences and surrounding Ad DNA, were apparently nondefective, and overtook the viral population a few passages after they were first detected. The results obtained from the analysis of these additional vectors seemed to confirm that as the net genome size decreased, the stability of the virus increased.

To rule out the possibility that differences in the level of expression of gB protein obtained with the gB expression vectors affected stability, the amounts of gB protein produced by AdABgB10, AdE1b$^-$gB10, and AdgB8 were compared by immunoprecipitation (data not shown). The levels of expression were similar for all three viruses and therefore did not appear to contribute significantly to the stability of the vectors. Because of the poor growth and rapid rearrangement of AdgB10, expression from this vector could not be analyzed.

DISCUSSION

In this study, the genetic stability of Ad5 vectors with net genome sizes ranging from 103.1 to 108.3% of the wt genome length was analyzed after serial passage in 293 cells. This range of genome sizes was obtained by constructing vectors with inserts of 4.88 (HSV-1 gB), 4.10 (HSV-1 gB), or 3.8 (LacZ) kb combined with a deletion in E3 of 1.88 or 2.69 kb. The results presented here show that the genetic stability of Ad5 vectors depends on net genome size. We have found that the larger the genome size over that of wt Ad5, the more genetically unstable the vector and the more rapidly it undergoes rearrangement.

Previous work has suggested that the Ad5 virion has the ability to package approximately 105% of the wt genome length, and this value is generally considered to be the maximum working capacity of the system (1, 15). Three of the largest genomes reported to date are found in Ad5in307 (30), Ad5in52 (15), and Ad5(pymT) (2), which have net genome sizes of 105.0, 105.7, and 106.0% of the wt Ad5 genome, respectively. AdgB10, the largest vector analyzed

in this study, exceeds 105% of the wt genome length by about 1.2 kb and was found to replicate much less efficiently than wt Ad5, requiring two to three times longer to induce a cytopathic effect following a typical infection of 293 cells. Analysis of viral DNA from serial passages of AdgB10 revealed that it was extremely unstable and underwent rearrangement within three to four passages (Fig. 1b and c). The other vectors analyzed in this study could be propagated with an efficiency indistinguishable from that of wt Ad5, even though they exhibited very different stabilities.

Vectors AdABgB10 (106.1%), AdgB8 (106.2%), and AdlacZ (105.4%), with net genome sizes much closer to 105% of the wt Ad5 genome, while more genetically stable than AdgB10, were still found to undergo rearrangement relatively quickly during serial passage. On average, rearrangements were first detected for these vectors by passage 9. Vectors AdE1b$^-$gB10 (104.6%) and AdAB14lacZ (103.1%), with net genome sizes below 105% of the wt genome length were significantly more stable. Rearrangement of AdAB14 lacZ was not detected until approximately passage 20 in the two series done with this vector, and rearrangement of AdE1b$^-$gB10 has not been detected to passage 20. These findings suggest that there is a relatively tight constraint on the amount of DNA which can be packaged into virions and that exceeding this limit results in sharply decreased yields of infectious virus. The packaging limit of SV40, a virus that, like Ad, has an icosahedral capsid, has also been reported to be approximately 105% (54). In a study similar to the one presented here, in which the packaging capacities of SV40 capsids were analyzed, similar results were observed in that the stability of the SV40 genomes correlated with net genome size (6). The tight packaging constraints seen for Ads and SV40 are likely due to the icosahedral structure of the capsid. In a report by Chang and Wilson (6), it is suggested that the packaging limit be thought of as a steep gradient, with the probability of being packaged becoming reduced with increased genome size. It is unclear whether the poor growth properties of viruses with oversized genomes are strictly due to the inability of larger genomes to be packaged or whether packaging of oversized viral genomes also results in physical instability of the virion, which would contribute to the poor growth properties of such viruses. Whatever the cause, the decreased growth rate, which was very obvious for AdgB10 but less apparent for the other vectors analyzed, presumably results in strong selection for variants which have undergone rearrangement, generating smaller genomes.

The rearrangements detected for the vectors analyzed in this study appeared to result in viable deletion variants that lost insert sequences and some surrounding Ad DNA in all cases, except for the three serial passages of AdABgB10 in which defective variants that could not replicate in the absence of helper functions provided by the parental virus were generated. In two of the serial passages of AdABgB10, variants that had lost essential viral sequences in *Hin*dIII fragment C (m.u. 17.4 to 32.2) arose, and in the third series (Fig. 7b), one variant lost insert sequences and presumably essential viral sequences surrounding the insert, while a second variant appeared to be viable and would likely have gone on to overtake the viral population in subsequent passages. The detection of rearrangements occurring in *Hin*dIII fragment C for AdABgB10, rather than in the insert, supports the idea that it is neither the specific insert sequences nor the context of the E3 substitution that causes genetic instability. AdgB10 and AdE1b$^-$gB10, which contain the same gB insert and flanking E3 sequences, exhibited very different stabilities, which could only be attributed to net genome size. It seems likely that in the viral population, variants with deletions in all areas of the genome arise but the selection for nondefective variants which can replicate more efficiently is very strong and therefore most of the variants we detected have lost nonessential insert sequences. In view of this, it is surprising that we did not detect variants with deletions in the E1 region, which is nonessential in these vectors because they were passaged in 293 cells which complement E1 functions.

The differences in stability observed for the vectors that express gB (AdABgB10, AdE1b$^-$gB10, and AdgB8) could not be attributed to differences in expression levels, since it was found that all three vectors expressed comparable amounts of gB. It does seem likely, however, that other vectors with sequences whose presence or expression is deleterious to viral replication could result in rapid selection for variants in which the insert has been deleted.

The most frequently used E3 deletion for vector construction is a 1.88-kb E3 deletion which, on the basis of the data presented here, should allow construction of stable vectors with inserts of 3.7 to 3.9 kb. Other E3 deletions which have also been utilized include a 1.65-kb deletion from 79.4 to 84.0 m.u. (11), a 2.29-kb deletion from 78.5 to 84.3 m.u. (49, 55), a 2.48-kb deletion from 78.8 to 85.7 m.u. (8), and a 3.0-kb deletion from 77.7 to 86.1 m.u. (44). In this report, we have described the construction of a number of plasmids with a 2.69-kb deletion in E3 that contain various restriction enzyme sites that facilitate the introduction of DNA inserts for rescue into infectious virus. This deletion, in addition to removing the major portion of all of the E3 messages, removed the first E3 splice acceptor site and the L4 polyadenylation site. The removal of these transcription signals did not appear to affect the growth kinetics (Fig. 4), progeny virus yield, or 100K expression levels (Fig. 5 and Table 1) significantly which suggests that removal of the L4 polyadenylation site did not seriously affect expression of the L4 products. This expanded E3 deletion should increase the working capacity of nonconditional helper-independent vectors to 4.5 to 4.7 kb.

The findings described in this report have important implications for the construction and use of vectors for expression, in live viral vaccines studies, and as gene transfer vectors for gene therapy. We found that although vectors with net genome sizes of approximately 105 to 106% of the wt Ad5 genome length can be easily generated, they can be genetically unstable and rearrange to generate variants with smaller genomes during propagation. Therefore, it may be prudent to limit the size of inserts as much as possible and to monitor carefully the structure of viral DNA for vectors whose genomes approach or exceed the size limits defined by these analyses.

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